A new description and association of a larva with the adult male of *Pliocaloca fidesria* Shackleton (Insecta: Trichoptera: Calocidae) from eastern Australia.

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Citation: Shackleton, M.E. & Webb, J.M. 2013 06 30. An association of a larva with the adult male of *Pliocaloca fidesria* Shackleton (Insecta: Trichoptera: Calocidae) from eastern Australia. *Memoirs of the Queensland Museum – Nature* 56(2): 593–600. Brisbane. ISSN 0079-8835. Accepted: 13 February 2013.

ABSTRACT

The genus *Pliocaloca* Neboiss consists of five species, of which the larva of only one, *P. kleithria* Shackleton, is known. Here we associate the larva of *P. fidesria* Shackleton. The larva is associated with adult males based on molecular and geographical data. Adult males and larvae were collected from the same location at Saddle Tree Creek, Bunya Mountains National Park (NP), southern Queensland. A 657 base pair (bp) fragment of the mitochondrial gene cytochrome oxidase sub unit 1 (COI) was used to verify species association. Two larvae and two adult males successfully yielded COI sequences. Sequences of the larvae and one of the adult males were identical. The other adult male differed from these by only one nucleotide. The larva of *P. fidesria* can be distinguished from *P. kleithria* by the presence of 9 setae in setal area 1 of the metanotum, the posterior margin of the pigmentation on the mesonotum being well defined, and the pigmentation of the metanotum being round. ❐ *Bunya Mountains National Park, Caddisfly, COI, idase, Larva, Pliocaloca, Taxonomy.*

Pliocaloca Neboiss, 1984 is an Australian endemic genus of caddisfly occurring in the northeast of the country. Neboiss (1984) established the genus initially with the description of three species from around the Cairns region: *P. fastigiata* Neboiss, 1984, *P. dasodes* Neboiss, 1984, and *P. mucronata* Neboiss, 1984. Jackson (1998 pp 12-13) provided a description of a larva of *Pliocaloca* (sp. AV1) in a preliminary key to the larvae of Calocidae Ross, 1967. She cited a pharate male pupa as the basis of this association and indicated that this undescribed species occurred in southern Queensland (QLD) and northern New South Wales (NSW). Shackleton (2010) described the adult males of *P. fidesria* Shackleton, 2010 from southern Queensland and the adult males, pupa, and larva of *P. kleithria* Shackleton, 2010 from northern New South Wales. At the time, it was not apparent if the larva illustrated in Jackson (1998) corresponded to the larva described in Shackleton (2010), but the setation and shape and size of sclerites of the mesonotum in *P. kleithria* appeared to differ from her illustrations. The larva Jackson used for these illustrations, along with her other Calocidae material, can no longer be located for comparative purposes.

Cartwright *et al.* (2013) noted that detailed larval descriptions were few for Australian taxa. In the current paper, the larva of *P. fidesria* is described and associated. This association reveals that the larval range for the *Pliocaloca*

sp. AV1 specimens in Jackson (1998) is a combined range of two species. Furthermore, the specimen illustrated in Jackson (1998) is likely to belong to *P. fidesria* as it shares diagnostic characters with this species.

Association of the larval and adult stages of trichopteran species has, in the past, primarily either been made through rearing larval specimens into adults, as in Drecktrah (1984), or examining the genitalic characters of pharate male pupae (Milne 1938). When pupae have not been able to give a reliable indication of association, some authors have inferred associations based on local occurrences, i.e. males, females, and larvae collected in the same location, as in Neboiss (1979). More recently, genetic data have been employed to associate life stages (Miller *et al.* 2005, Zhou *et al.* 2007).

Here morphological characters and population segregation are used to infer species delimitation. This inference is tested using sequences of the mitochondrial gene cytochrome oxidase sub unit 1 (COI). Association is inferred through sequence homology of the COI gene within the species and supported by the occurrence of larvae and adults existing together at the same site. This association will enhance the capabilities of studies, such as river health monitoring programs, which depend on accurate identifications of larval specimens.

MATERIALS AND METHODS

All material was collected into 100% ethanol and is deposited in the Queensland Museum, Brisbane (QM). Shackleton (2010) was used to identify the adult specimens and the keys of Jackson (1998) and Shackleton (2013) to identify the larval specimens. Material was examined using a Leica MZ16 stereo microscope. Photographs were taken using a Leica DFC320 camera mounted on a Leica MZ16 microscope. Photographs were edited using GIMP 2.6.11.

A small amount of tissue (usually a leg) was taken from each specimen and used to obtain a 657 base pair (bp) fragment of the mitochondrial gene cytochrome oxidase sub unit 1 (COI). Methods for extraction and amplification of this gene fragment are detailed below.

Extraction method. Deoxyribonucleic acid was extracted using a 5% Chelex solution and Proteinase K mix. A 657 base pair (bp) fragment of the mitochondrial gene cytochrome oxidase sub unit 1 (COI) was amplified. For all specimens of *P. fidesria* and 3 specimens of *P. kleitlria*, DNA extraction, amplification, and sequencing were conducted at the Canadian Centre For DNA Barcoding (CCDB, Guelph, Ontario, Canada). Protocols used by the CCDB are available at www.dnabarcoding.ca. For the remaining specimens, DNA extraction and amplification were performed at La Trobe University (LTU) (Wodonga, Australia). Sequencing of these specimens was conducted by Macrogen Inc (Seoul, Korea). The LTU methods consisted of amplifying COI using the primers LCO1490 and HCO2198 (Folmer *et al.* 1994); primers were M13-tailed to facilitate sequencing. The Polymerase Chain Reaction (PCR) cocktail for reactions consisted of 4µl buffer, 20µl 10% w/v trehalose, 0.8µl deoxynucleotide triphosphates (dNTPs), 2µl 50mM $MgCl_2$, 0.8µl of each primer, 0.1µl taq polymerase (Invitrogen), 0.01-5µl of DNA template, and water to 40µl. PCR conditions consisted of 1 min at 94° C; 5 cycles of 1 min at 94° C, 1.5 min at 45 °C, 1.5 min at 72° C; 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72° C; and 4 min at 72° C.

For the resultant data, contigs were assembled in DNABaser 2.75 (www.DnaBaser.com) and aligned with default settings in Clustal X as implemented in MEGA5 (Tamura *et al.* 2011). All sequences were examined for the presence of double peaks, frame shifts, and stop codons. Sequences were submitted to GenBank under the accession numbers given in Table 1. A search for similar sequences on GenBank was conducted using the Basic Local Alignment Search Tool (BLAST) and the resultant sequences were added to the data set.

A sequence of *Tamasia variegata* Mosely, 1936 was added to the data set as an outgroup. The data set was partitioned into three categories, according to the position of base pairs with the codon (COI1, COI2, COI3), and the best

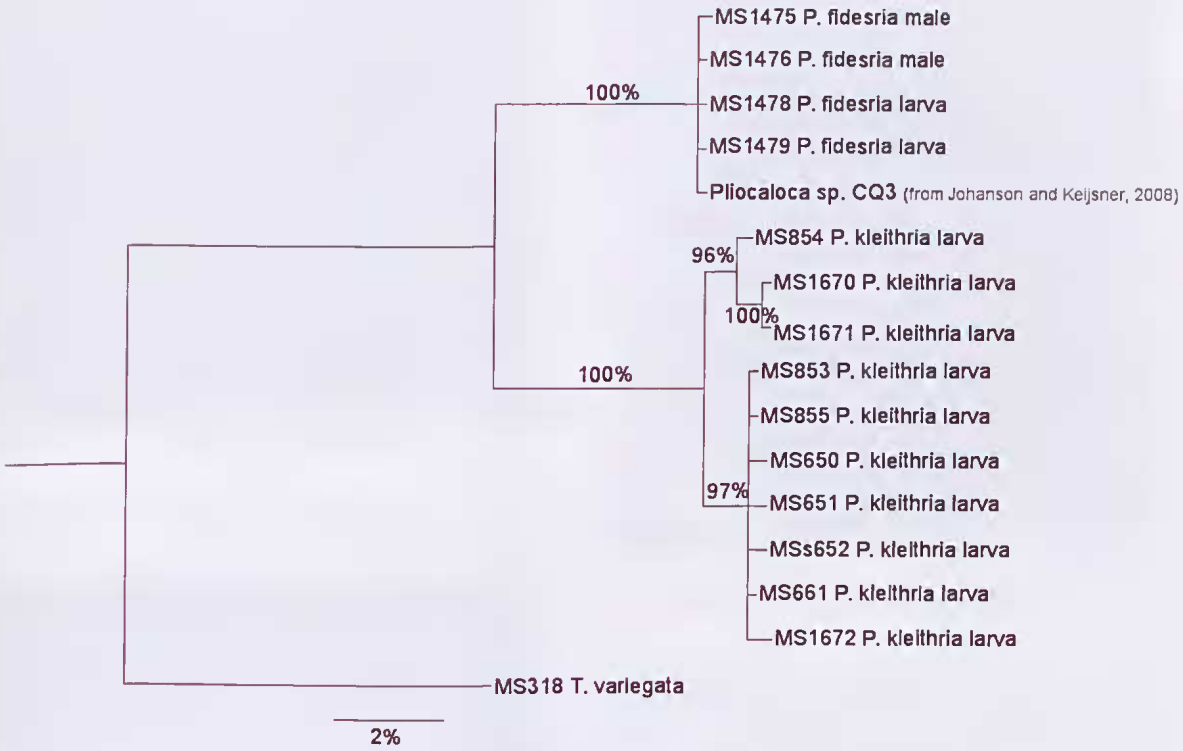

FIG. 1. Bayesian inference of 657 bp barcoding region of cytochrome oxidase subunit 1 including 4 specimens of *P. fidesria*, 8 specimens of *P. kleithria*, and an outgroup specimen of *T. variegata*. Posterior probabilities are indicated on branches. Scale bar represents percent divergence.

model of evolution was determined for each partition using MrModeltest (Nylander 2004) and Phylogenetic Analysis Using Parsimony (PAUP) (Swofford 1999). Evolutionary models were selected from the Akaike Information Criterion (AIC) given in the MrModeltest (Nylander 2004) outputs. A Bayesian analysis was performed using MrBayes 3.1 (Ronquist & Huelsenbeck 2003), in which ten million generations were run and a sample taken every one thousand generations. The first 25% (2500) of the trees generated were deleted from the statistical summary as a 'burn in'. The resultant tree (Fig. 1) was rooted using the sequence of *T. variegata*. Pairwise p-distances were calculated in MEGA5 (Tamura *et al.* 2011) and were used to calculate minimum inter-specific divergence and the maximum intra-specific divergence. Specimens collected for this study and used for molecular analysis are indicated by the accession numbers of the senior author with the prefix 'MS'. The sole sequence obtained through GenBank is indicated by the accession number assigned by the author of that sequence followed by the publication in which the sequence was first published.

RESULTS

Four specimens of *Pliocaloca fidesria* (two males and two larvae) successfully yielded 657 bp long COI sequences. The BLAST search retrieved one sequence from an undescribed *Pliocaloca* species (sp. CQ3), with 90% query coverage and 99% percent similarity. The sequence was originally published in Johanson and Keijsner (2008). For all specimens of *P. fidesria* the COI sequences were identical except that in specimen MS1475 a thiamine was present at position 216 as opposed to a cytosine in the

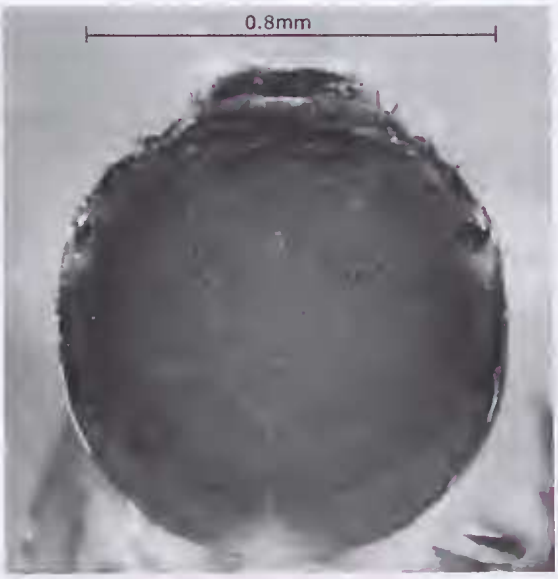

FIG. 2. *Pliocaloca fidesria* Shackleton. Larva, dorsal views. Head.

FIG. 3. *Pliocaloca fidesria* Shackleton. Larva, dorsal view pronotum.

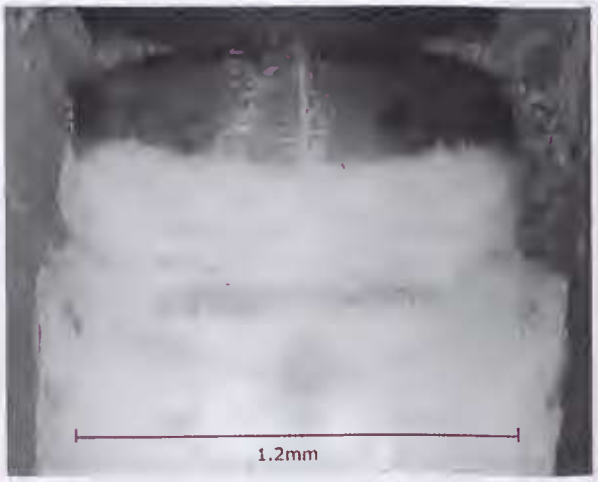

FIG. 4. *Pliocaloca fidesria* Shackleton. Larva, dorsal view, mesonotum and metanotum.

other specimens. Sequences of COI were also obtained from 10 larval specimens of *P. kleithria*. The maximum p-distance, calculated using pairwise deletion, within *P. fidesria* was 0.17% and within *P. kleithria* was 2.85%. The minimum distance between the two species was 6.76%.

The AIC returned the following models: GTR for first codon position, HKY for second codon position, and HKY+I for third codon position. After ten million generations in MrBayes 3.1 (Ronquist and Huelsenbeck 2003), the standard deviations of split frequencies fell below 0.01, stationarity was assumed to have been reached, and the analysis was stopped. The resultant tree (Fig. 1) indicated a large distance between the two *Pliocaloca* species with 100% posterior probability support for the separation of the two species. Three larval specimens of *P. kleithria* occurred as a sister clade to the rest of the *P. kleithria* specimens. The specimens of *P. fidesria* formed a monophyletic clade with 100% posterior probability support.

DISCUSSION

Analysis of the COI gene indicates that the *Pliocaloca* larvae found at Saddle Tree Creek, Bunya Mountains National Park, belong to the species *P. fidesria*. The larvae shared an identical COI sequence with one of the males collected and differed by only one base pair from the other male adult. It is unlikely that our result is a product of a shared ancestral polymorphism between two species, as recent studies suggest that COI sequences shared between species is rare. Hebert *et al.* (2009) found only 9 cases of shared COI sequences between 1327 species of Lepidoptera, and then

TABLE 1. Specimen details for sequenced specimens including GenBank, BOLD, and La Trobe University (LTU) accession numbers and processing institutions. CCDB=Canadian Centre for DNA Barcoding.

Species	Life stage	Locality	Date collected and collector	LTU accession number	COI GenBank reference	Amplification and sequencing institute	BOLD specimen id
Pliocaloca fidesria	male	Saddle tree creek, Bunya Mtns NP, southern QLD	23 Nov 2011 J. Mynott and M. Shackleton	MS1475	KC860488	CCDB	LTUT470-11
Pliocaloca fidesria	male	Saddle tree creek, Bunya Mtns NP, southern QLD	23 Nov 2011 J. Mynott and M. Shackleton	MS1476	KC860491	CCDB	LTUT471-11
Pliocaloca fidesria	larva	Saddle tree creek, Bunya Mtns NP, southern QLD	23 Nov 2011 J. Mynott and M. Shackleton	MS1478	KC860490	CCDB	LTUT473-11
Pliocaloca fidesria	larva	Saddle tree creek, Bunya Mtns NP, southern QLD	23 Nov 2011 J. Mynott and M. Shackleton	MS1479	KC860489	CCDB	LTUT474-11
Pliocaloca kleithria	larva	Coppernook ck, Dorrigo NP, northern NSW	10 Nov 2011 J. Mynott and M. Shackleton	MS836	KC860498	LTU / Macrogen	
Pliocaloca kleithria	larva	Tributary Wilson River, 70m along Falls walk track, Willi Willi NP, northern NSW	04 Dec 2007 A. Glaister, J. Dean, and R. St. Clair	MS650	KC860496	LTU / Macrogen	
Pliocaloca kleithria	larva	Tributary Wilson River, 70m along Falls walk track, Willi Willi NP, northern NSW	04 Dec 2007 A. Glaister, J. Dean, and R. St. Clair	MS651	KC860495	LTU / Macrogen	
Pliocaloca kleithria	larva	Tributary Wilson River, 70m along Falls walk track, Willi Willi NP, northern NSW	04 Dec 2007 A. Glaister, J. Dean, and R. St. Clair	MS652	KC860494	LTU / Macrogen	
Pliocaloca kleithria	larva	Eight mile ck, Bullock rd, northern NSW	04 May 2010 NSW Department of Environment and Climate Change	MS661	KC860487	LTU / Macrogen	
Pliocaloca kleithria	larva	Williams River at rest area beneath 1st bridge along walking track, Barrington Tops NP, northern NSW	09 Nov 2011 J. Mynott and M. Shackleton	MS853	KC860493	CCDB	LTUT035-11
Pliocaloca kleithria	larva	Williams River at rest area beneath 1st bridge along walking track, Barrington Tops NP, northern NSW	09 Nov 2011 J. Mynott and M. Shackleton	MS854	KC860500	CCDB	LTUT036-11
Pliocaloca kleithria	larva	Williams River at rest area beneath 1st bridge along walking track, Barrington Tops NP, northern NSW	09 Nov 2011 J. Mynott and M. Shackleton	MS855 .	KC860499	CCDB	LTUT037-11
Pliocaloca kleithria	larva	Creek at waterfall above Darraboola falls, Lamington National Park, QLD	17 Nov 2011 J. Mynott and M. Shackleton	MS1670	KC860492	La Trobe / Macrogen	
Pliocaloca kleithria	larva	Creek at waterfall above Darraboola falls, Lamington National Park, QLD	17 Nov 2011 J. Mynott and M. Shackleton	MS1671	KC860497	La Trobe / Macrogen	
Tamasia variegata	larva	Unnamed River on Racecourse trail @ brushy mt picnic area, Werrikimbe NP, northern NSW	12 Nov 2011 J. Mynott and M. Shackleton	MS823	KC860501	La Trobe / Macrogen	

only between congeneric and morphologically similar species. Webb *et al.* (2012) found no shared COI sequences between Ephemeroptera species of Northern America. Among studies involving caddisflies, Hogg *et al.* (2009) found no sequences shared between 61 New Zealand species and Zhou *et al.* (2011) found no shared COI sequences between 209 species from the Great Smoky Mountains National Park, USA.

In the past, association of these two forms would have been made based on the occurrence of the two forms occurring at the same site with no other similar species occurring close to the site. *Pliocaloca fidesria* appears to be an isolated species with no other *Pliocaloca* species existing within its range. The range of its closest neighbouring species is not known to extend within around 300 km from *P. fidesria*. Furthermore, the larvae and adults, collected for this project, were collected from the same site. This indicates that *P. fidesria* is a candidate for an association based on geographic data alone. However, the COI data also provide strong evidence in support of this association. The present study indicates that analysis of molecular data alone may serve as a reliable method for associating life stages in other species. This method is particularly useful when pharate male pupae, which have traditionally used to infer associations, but are difficult to collect or rear out, are not available.

The *P.* sp. AV1 larva depicted in Jackson (1998, figs. 1.12–1.17) resembles the larva of *P. fidesria*. Her illustration (fig. 1.16) clearly shows that the pigmentation on the metanotum has a strongly demarcated and relatively straight posterior margin. Also, on the metanotum the pigmentation patch is rounded and 9 pairs of setae are present anteriorly on the segment. These characters are all present on our larvae of *P. fidesria* associated with male adults. Given that only two species are known to exist in the northern NSW and southern QLD region (Shackleton 2013) it is likely that the *Pliocaloca* sp. AV1 larva depicted in Jackson (1998) belongs to *P. fidesria*.

The findings here provide the ability to place a species identity to larval specimens of *P. fidesria*, which is important for those conducting river health monitoring programs and other scientific enquiries involving larval specimens. Furthermore, it allows researchers to distinguish between the larvae of *P. fidesria* and *P. kleithria*, which has, until now, not been possible.

SYSTEMATICS

Family CALOCIDAE

Pliocaloca Neboiss, 1984

Type species. *Pliocaloca mucronata* Neboiss, 1984, by original designation, from northern Queensland.

Pliocaloca fidesria Shackleton

Material examined. Queensland. Saddle Tree Creek at Festoon Falls. Bunya Mountains National Park (-26.848611S, 151.56166E) 23 November 2011: MS1475 1 male (QM – T183456); MS1476 1 male (QM – T183457); MS1478 1 larva (QM – T183458); MS1479 1 larva (QM – T183459); MS1480 1 larva (QM – T183460); MS1481 1 larva (QM – T183461).

Diagnosis. *Pliocaloca* larvae are distinguishable from other Calocidae larvae in that the setae of the head are large and flattened; the pronotum is covered in short, dense, papillose setae; and the metanotum has a pigmented patch anteriorly, which is not raised. In *Pliocaloca* and *Calocoides* Neboiss, but no other Calocidae, the foretrochantin is not fused to the propleuron. The larva of *P. fidesria* differs from *P. kleithria* in that the pigmentation of the mesonotum is more defined along the posterior margin; in *P. kleithria* this posterior margin is encroached by unpigmented areas, especially along the midline where the unpigmented area extends anteriorly about half way into the pigmentation. In the metanotum of *P. fidesria* setal area 1 contains about 9 setae as opposed to *P. kleithria* which has around 6 setae. The pigmented patch on the metanotum appears more rounded in *P. fidesria* than in *P. kleithria*, which is somewhat more elongate and pointed towards the posterior (see Shackleton 2010, Fig. 23). In the original description of *P. kleithria* (Shackleton 2010) the pigmented patch on the metanotum is incorrectly described as being rounded.

Description. *Male*: as described in Shackleton (2010).

Female. Unknown.

Pupa. Unknown

Larva. Length: 9.4-9.9 mm. Head (Fig. 2): dorsum papillate, with conspicuous, mesially directed, flattened setae; antennae situated half way between eye and anterior margin of head capsule. Pronotum (Fig. 3): dorsum covered with spinules, with dense, short setae; anterior margin with dense, short setae; each sclerite with anterior margin curved forward between medial suture and lateral margin; lateral carina present, not extending to dorsum, with fringe of setae on dorsal margin; foretrochantin not fused to propleuron. Metanotum (Fig.4): posterior margin of pigmentation relatively straight and well defined. Mesonotum (Fig.4): setal area 1 with about 9 setae closely spaced; pigmentation patch rounded. Abdomen: abdominal segment I spiny patch present without lateral sclerite; abdominal gills absent; lateral sclerite of segment X with many setae; anal claws each with 1 accessory tooth. Case: curved cylinder of sand grains, posterior aperture round.

ACKNOWLEDGEMENTS

Julia Mynott assisted with collecting specimens. Material from Queensland was collected under permit WITK10277111. Material from New South Wales was collected under Scientific Research Permit number P07/0095 and National Parks Service Scientific Licence number S13223. This project was conducted as part of the Taxonomic Research and Information Network (TRIN) and was funded by the Commonwealth Environment Research Facilities (CERF) program. We are grateful for support provided by the Government of Canada through Genome Canada and the Ontario Genomics Institute (2008-OGI-ICI-03) to the International Barcode of Life Project. This funding enabled the Canadian Centre for DNA Barcoding (University of Guelph) to carry out the sequence analysis on our specimens. We also thank the Ontario Ministry of Economic Development and Innovation for funding the ongoing development of BOLD.

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